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Human Mitochondrial 3,2-*trans*-Enoyl-CoA Isomerase (DCI): Gene Structure and Localization to Chromosome 16p13.3

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A key enzyme in the mitochondrial β-oxidation of unsaturated fatty acids is the 3,2-*trans*-enoyl-CoA isomerase (DCI; EC 5.3.3.8). It catalyzes the transformation of 3-*cis* and 3-*trans* intermediates arising during the stepwise degradation of all *cis*-, mono-, and polyunsaturated fatty acids to the 2-*trans*-enoyl-CoA intermediates. A genomic clone encoding the human DCI was isolated and characterized by use of the previously cloned human DCI cDNA. The entire gene encompasses approximately 12.5 kb, and the coding sequence is distributed over seven exons. One major and three minor transcription start sites were determined by primer extension analysis. In common with promoters of other housekeeping genes encoding mitochondrial proteins, the GC-rich, immediate 5′-flanking region of the DCI transcription initiation site lacks typical TATA and CAAT boxes; instead, two GC box consensus sequences are present. Introns 2 and 6 contain several *Alu* repetetive sequences. The human DCI gene locus was assigned to chromosome 16 by use of human–rodent somatic cell hybrids and to chromosome 16p13.3 by chromosomal *in situ* suppression hybridization studies. © 1994 Academic Press, Inc.

INTRODUCTION

Mitochondrial β-oxidation of unsaturated fatty acids leads to intermediates not compatible with the conventional set of the four enzymes responsible for the degradation of saturated fatty acyl-CoA esters (Stoffel, 1966). The further processing of these intermediates requires additional enzymes, depending on the position of the double bonds in the original fatty acids. Oxidative degradation of unsaturated fatty acids with double bonds at odd-numbered positions yields 3-*cis*-enoyl-CoA esters, whereas those having double bonds at even-numbered positions are chain-shortened to 2-*cis*-enoyl-CoAs, hydrated to the D-(−)-3-hydroxy derivatives, and epimerized to the L-(+) intermediates or converted by the 2,4-dienoyl-CoA reductase (EC 1.3.1.34) to 3-*trans*-enoyl-CoAs. The reentry of the 3-*cis*- and 3-*trans*-enoyl-CoA esters into the β-oxidation spiral is accomplished by the mitochondrial 3,2-*trans*-enoyl-CoA isomerase (DCI[2]; EC 5.3.3.8). This enzyme catalyzes the shift of the double bonds of 3-*cis*- as well as 3-*trans*-isomers to the 2-*trans*-enoyl-CoAs, known as regular substrates of the 2-*trans*-enoyl-CoA hydratase (EC 4.2.1.17).

Since the discovery of the mitochondrial DCI (Stoffel *et al.*, 1964a,b), the enzyme has been purified from the liver tissue of different mammalian species (Euler-Bertram and Stoffel, 1990; Stoffel and Grol, 1978). Rat liver DCI cDNAs have recently been cloned and sequenced (Müller-Newen and Stoffel, 1991; Palosaari *et al.*, 1991). The open reading frame encodes the 289-amino-acid residue polypeptide of the preisomerase. Using the rat DCI cDNA as hybridization probe, we were at last able to clone and sequence a human DCI cDNA encoding the cytosolic preform of the human DCI with a cDNA-derived amino acid sequence of 302 residues (submitted for publication).

Significant sequence homologies between rat and human DCIs, the amino-terminal half of the rat peroxisomal multifunctional isomerase-hydratase-dehydrogenase enzyme (Palosaari and Hiltunen, 1990), the α-subunit of the prokaryotic β-oxidation complex encoded by the fadB operon of *Escherichia coli* (DiRusso, 1990), and the rat mitochondrial enoyl-CoA hydratase (Minami-Ishii *et al.*, 1989), suggest a common evolutionary origin of these functionally related enzymes.

Inborn errors causing metabolic diseases of mitochondrial fatty acid oxidation have only recently been described as human genetic defects (Coates and Tanaka, 1992). Most commonly under fasting conditions, impairments of this important energy-providing pathway are associated with different severe clinical findings, especially for affected infants or young children. As many as 12 individual inherited human disorders

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[2] In accordance with the HGMW nomenclature for human genes, we have designated the human gene encoding mitochondrial 3,2-*trans*-enoyl-CoA isomerase DCI (based on its U.B. name dodecenoyl-coenzyme A Δ-isomerase).

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of mitochondrial fatty acid oxidation have been reported to the present. Nevertheless, there are numerous additional clinical observations referring to an altered fatty acid oxidation, none of which is related to any of the known defects. Thus, it is likely that various genetic disorders of this pathway are still unidentified.

An essential prerequisite for the elucidation of metabolic diseases on the basis of genetic defects of β-oxidation enzymes is the characterization of their gene structure and chromosome assignment.

MATERIALS AND METHODS

Isolation of genomic DCI clones. The entire ^{32}P-labeled (Feinberg and Vogelstein, 1984) human DCI cDNA derived from phDCIc-1 (1017 bp, *Eco*RI digested) served as hybridization probe for screening a human placental genomic DNA library (Product No. HL1067j, Clontech), ligated into the *Bam*HI site of the λ phage EMBL-3 SP6/T7. Screening of approximately 5×10^5 phage plaques yielded four positive clones, denoted λhDCIg-*X* (*X* = 1–4). Phage DNA of the four clones was prepared by established procedures (Maniatis *et al.*, 1982), sizes of the genomic inserts were estimated, and the inserts were characterized by Southern blotting with the 5′-end-labeled oligonucleotides Iso30Ns 5′-ATGGCGCTGGTGGCTTCTGTGCGAGTCCCG-3′ and Iso30Cas 5′-TTAGCCTTTTTCTTCTTTGAGCCTCTCTAA-3′ representing the 5′- and 3′-end of the coding region of the DCI cDNA. The 18-kb insert of clone λhDCIg-2—proved to harbor the entire coding sequence—was subjected to further characterization.

Restriction analysis and subcloning of phage DNA. The genomic insert of phage clone λhDCIg-2 was partially mapped by single and double restriction digestion with *Bam*HI, *Sac*I, *Sfi*I, *Xba*I, and *Xho*I, followed by Southern blot hybridization using appropriate ^{32}P-labeled oligonucleotides as probes. Genomic fragments encompassing exon sequences and the 5′-flanking region were subcloned into the multiple cloning site of the vector pBluescript SK(+) (Stratagene). The 8-kb *Xho*I fragment (phDCIg-8) was further digested with *Kpn*I, and the resulting fragments, 4.8, 1.3, and 1.9 kb in size, were ligated into the same vector and designated phDCIg-8a, -8b, and -8c, respectively. Sequencing of the genomic subclones was carried out by the dideoxy chain termination method (Sanger *et al.*, 1977) using the T7-sequencing kit (Pharmacia) with M13, M13 reverse primers, and synthetic oligonucleotides designed from established sequences. Primer pairs for determination of intron sizes by polymerase chain reaction (PCR) were selected for amplification of DNA either between exons or between the T3/T7 promoter and exons in appropriate subclones. The PCR conditions were as follows: denaturation step at 94°C for 3 min; 30 cycles each at 93°C for 1.5 min, 45°C for 2 min, 72°C for 2 min; and a final amplification step at 72°C for 7 min in a Perkin-Elmer Cetus thermocycler.

Primer extension. The transcription start site was determined by primer extension using the synthetic antisense oligonucleotide ISO30Nas 5′-CGGGACTCGCACAGAAGCCACCAGCGCCAT-3′ representing the first 30 nucleotides of the coding region. It was 5′-labeled with [γ-^{32}P]ATP and polynucleotide kinase and then hybridized to 6 μg of human liver poly(A)$^+$ RNA. The primer extension reaction was performed following the standard procedure (McKnight and Kingsbury, 1982) with Moloney murine leukemia virus RNaseH$^-$ reverse transcriptase (GIBCO-BRL). Extension products were analyzed on a 7 *M* urea, 5% polyacrylamide gel adjacent to a sequence ladder generated with the same 30-bp oligonucleotide used as primer for reverse transcription.

Chromosomal assignment of human DCI. The sense primer IsoC-1s 5′-GACCAGCAGCACCTAAGAGCCCTGTCCGGC-3′ complementary to a sequence of intron 4 and the antisense primer IsoC-2as 5′-AGAAAACCTTTGGGTGGAGAACCTTCTGGT-3′ complementary to a sequence of intron 6 of the human DCI gene were used for PCR of human/rodent somatic cell hybrid DNA with individual chromosomes and of human, mouse, and Chinese hamster total DNA (1 μg DNA template) as controls (NIGHS human/rodent somatic cell hybrid panel 2). Standard Mg^{2+}, dNTP, primer concentrations, and the following conditions were applied: samples with a total volume of 30 μl were initially denatured at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min. A Perkin-Elmer 9600 PCR machine was used. Fifteen microliters of the reaction mix was analyzed on a 2% agarose gel; *Msp*I-restricted pBR322 was used as marker.

Mapping of the human DCI to chromosome 16p13.3. Total DNA from the plasmid phDCIg-8 was labeled by nick-translation using digoxigenin–11-dUTP (Kessler *et al.*, 1990) (Boehringer Mannheim) or biotin–11-dUTP (Langer *et al.*, 1981) (Sigma). This probe was used for chromosomal *in situ* suppression (CISS) hybridization to elongated metaphase chromosomes as described (Lichter *et al.*, 1988, 1990). Briefly, 150–200 ng labeled probe was combined with 10 μg Cot1 DNA, ethanol precipitated, and finally resuspended in 10 μl hybridization cocktail (50% formamide, 10% dextran sulfate, 2× SSC). Following heat denaturation at 75°C for 5 min, preannealing of probe and competitor DNA was carried out at 37°C for 30 min. Hybridization signals were recorded with a Zeiss Axioplan microscope equipped for conventional epifluorescence microscopy using a CCD camera (Photometrics). FITC and DAPI images were overlayed electronically, and photographs were taken directly from the video screen.

In situ hybridization. Chromosome preparations were obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 h according to standard protocols. Denaturation of the slides at 70°C for 2 min in 70% deionized formamide, 2× SSC was followed by dehydration in ice-cold ethanol. Hybridization at 37°C and posthybridization washes are described elsewhere (Lichter *et al.*, 1988). The biotin- or digoxigenin-labeled probe was detected by incubation with avidin conjugated to fluorescein isothiocyanate (FITC) (Vector Laboratories) or antidigoxigenin conjugated to rhodamine (Boehringer), respectively. Counterstaining with 4,5-diamidino-2-phenylindol-dihydrochloride (DAPI) was performed to confirm the identity of the chromosomes.

RESULTS

Structure and Organization of the Human DCI Gene

Using the human DCI cDNA (Fig. 1a) as hybridization probe, four positive clones have been isolated from a human placental genomic library. Restriction mapping and Southern blot hybridization analysis with specific oligonucleotides derived from the cDNA revealed that the 18-kb insert of clone λhDCIg-2 encompasses the entire DCI coding region. This genomic insert was cleaved by restriction enzymes into appropriate fragments (Fig. 1c), which were subcloned for sequence analysis of the 5′-untranslated region, the exons, the exon/intron junctions, and approximately 4 kb of intron sequences. Alignment of the human DCI cDNA with the established genomic sequences indicated that the DCI gene covers 12.5 kb of the human genome and consists of seven exons separated by six introns, as depicted in Fig. 1b. The 5′- and 3′-UTRs are included in exon I and exon VII, respectively. All exon/intron boundaries are in agreement with the consensus sequence around splice sites (Breathnach and Chambon, 1981). The organization and sizes of each exon, the exon/intron boundary sequences, and the intron sizes are summarized in Table 1. The exon sequences encompass approximately 9% of the gene. They agree with

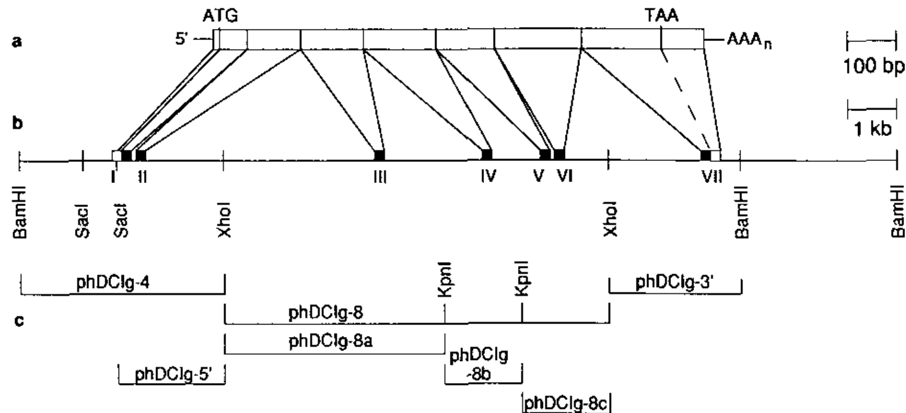

FIG. 1. Structure of the human DCI gene. (**a**) Human DCI mRNA. (**b**) Restriction map of the 18-kb genomic insert of phage clone λhDCIg-2 containing the entire DCI gene. The exons, numbered I–VII, and the introns are represented by filled boxes and lines, respectively. The untranslated regions of exon I and VII are designated by open boxes. (**c**) Subclones used for sequencing.

the human DCI cDNA except one silent G to C change in codon 16. Two *Alu*-like sequences have been identified within intron 2 and five within intron 6.

Identification of Transcription Initiation Site

Human liver poly(A)$^+$ RNA was used for the determination of the transcription start site of the DCI gene. The untranslated region was extended from the 5′-end-labeled oligonucleotide Iso30Nas encoding the 10 N-terminal amino acid residues of the signal sequence. The extension products were analyzed on a sequencing gel by alignment to a sequence ladder generated by the dideoxy chain termination reaction of subclone phDCIg-4, also using Iso30Nas as primer. Heterologous transcripts were obtained, as shown in Fig. 2. The major band, however, corresponded to nucleotide T (71 bp upstream of the translation start codon) and was designated the transcription start in the genomic DNA sequence (nucleotide position +1).

Analysis of the 5′-Flanking Region

The nucleotide sequence of an additional 688 bp upstream of the major transcription initiation site of the human DCI gene was analyzed using a software program (Genetics Computer Group, 1991) that identified known regulatory sequences (Fig. 3). The analysis revealed that the immediate upstream region of the transcription initiation site is GC rich (≈72%). It lacks typical TATA or CAAT box sequences. Instead, the putative promoter region contains two Sp-1 binding motifs (GC boxes, GGGCGG) between positions −2 and −11 and −63 and −68 upstream of the translation start. In addition, a CTF/NF1 binding site (−114 to −125), the phorbolester responsive element AP-1 (−397 to −403), and an AP-2 binding site (−342 to −349) could be identified.

Chromosome Localization of the Human DCI Gene

We assigned the DCI gene locus to chromosome 16 by PCR using a sense oligonucleotide homologous to an intron 4 sequence and an antisense primer homologous to an intron 6 sequence and DNA of the NIGMS human/rodent somatic cell hybrid panel with individual chromosomes of human, mouse, and hamster as template (Fig. 4a). To localize the gene for the human DCI more precisely, we used the plasmid phDCIg-8 for fluorescence *in situ* hybridization to elongated metaphase chromosomes (Fig. 4b). Because of repetitive sequences within the 8-kb insert of phDCIg-8, it was necessary to carry out preannealing with Cot1 DNA for 30 min.

TABLE 1

Exon/Intron Sizes and Sequences at Splice Junctions of the Human DCI Gene

Exon	Size (bp)	Exon/intron junction sequences: 5′-Donor site	Exon/intron junction sequences: 3′-Acceptor site	Intron	Size (bp)	Amino acids interrupted
I	≥123	CGG GCG G gtgcga	gccttcgcag GG GCC CGG	1	85	Gly^{18}
II	114	GCC GCA G gtgagc	tttaaattag GG GTC GCT	2	≈5000	Gly^{56}
III	128	CTG ACC TCG gtaggt	tcccctgcag GAC CGC CCG	3	≈2100	Ser^{98}/Asp^{99}
IV	147	GCC ATC AAC gtgagt	ctccctgcag GGA GCC TGC	4	970	Asn^{147}/Gly^{148}
V	122	CCT TTC TG gtaagg	gcatttccag G TTG AAA	5	93	Trp^{188}
VI	179	ATT CCA G gtgagg	ttctctttag AC CAT GCT	6	≈3100	Asp^{248}
VII	≥251					
Consensus splice signal		NAG gt$^{a}_{g}$agt	$^{cccccc}_{tttttt}$n$^{c}_{t}$ag G			

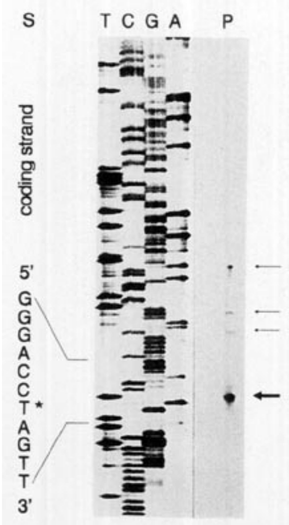

FIG. 2. Transcription start determined by primer extension of DCI mRNA. Six micrograms of human liver poly(A)$^+$ RNA was subjected to primer extension analysis with the 5'-end-labeled 30-bp oligomer Iso30Nas complementing the sequence encoding the amino terminus of human DCI. The sequence ladder (lanes **T**, **C**, **G**, and **A**) was obtained by sequencing the 5'-flanking region (phDCIg-4) with Iso30Nas. Lane **P**, primer extension products marked by arrows. The nucleotide identified as the major transcription start site is depicted by an asterisk (lane **S**).

Hybridization of biotinylated or digoxigenin-labeled plasmid DNA and detection via FITC or rhodamine, respectively, resulted in specific signals in the distal region of the short arm of chromosome 16. From one experiment, 26 of 35 metaphase chromosomes showed specific signals on both homologues in this region. This specific labeling is apparent despite weak background hybridization to other chromosomes, which, however, did not appear consistently on any particular chromosomal band. DAPI staining of metaphase chromosomes revealed that the cytological position of the gene for the human DCI is 16p13.3.

DISCUSSION

The present paper describes the cloning and characterization of the human DCI gene. The gene covers 12.5 kb of human chromosome 16 DNA at band p13.3 and consists of seven exons and six introns. Except for one silent G to C change in codon 16 there is perfect agreement between the coding region of the gene and the previously determined DCI cDNA. The heterologous transcription start sites determined by primer extension analysis may be explained by the lack of typical TATA and CAAT boxes in the putative promoter region of the DCI gene. Like promoters of other housekeeping genes, the immediate 5'-flanking region of the human DCI gene is rich in GC bases and contains two GC boxes (Dynan, 1986) that may be responsible for the RNA polymerase II binding to the TATA-box free promoter and thereby for transcription initiation and effective transcription (Blake *et al.,* 1990). Whether the transcription factor-binding sites found in the 5'-flanking region are involved in the regulation of DCI gene

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-688  tgcttaagat gagaacaaaa gttgagacca cccaacacag ctgggacctt actgcccagt  -629

-628  gctcccagtg gtctgacgaa aaccccacac cagacagtac aaaggcccac tcccgagctg  -569

-568  ccgcttacgg cccagcggga acagactgta ttcttatctt tcggcatttc tctgcaaata  -509

-508  aaaaggtgcc actgcggtcc ggcctttgtg cacttgccct tgtgctgagc aaggcgggtg  -449
                                                     AP-1
-448  gcctgttctt accgctgggt tctgctgccc ccaccccat ttcgg[tgact aa]acctgcag  -389
                                                 AP-2
-388  ctctgatcct gttcccaaag gggcaggagg ccgggctca[c cccaggc]agc ccctggcctc  -329

-328  ctgacggccc cctcgggagg ggttccgtgc tttgatcgcc tttccagcgc cgatactgca  -269

-268  gtagctgggc ccggccgaga gctcgcggtc acgaacaccg gcagcctctt gcctacagtg  -209

-208  tccgcgctgc agaagagcct gccctggcgc tgagctggtc gtggctgctt cagcagtggc  -149
                                     CTF/NF1
-148  ctgcgcctcc ttgtagccag ccc[tggccca gatcc]aggtg cctgccccgg aagcgcgcgg  -89
                             SP-1
-88   ctgcccgggg tgaagccgga [gggcgg]cgag cggctgtttc ggccggtagg tggaggcgtt  -29
                 *     *     SP-1     +1
-28   gaccagcctc tgggaac[ggg gcgggac]cTA GTTGCGTGCC GCCCCCTCCC CAGGAGGCGA  32

33    GCTCAGCCCG CGACCTTTAT CCCGCGCGTT GCGGTCAAGA TGGCGCTGGT GGCTTCTGTG  92

93    CGAGTCCCGG CGCGCGTTCT GCTCCGGGCG Ggtgcg                              128
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FIG. 3. Nucleotide sequence of the 5'-flanking region of the DCI gene. The major transcription start site is indicated by a boldface letter (nucleotide T, +1). Three minor start sites are marked by asterisks. The 5'-untranslated region and the subsequent coding region (ATG, underlined) are in capital letters. Putative transcriptional regulatory elements, the binding motifs of SP-1, CTF/NF1, AP-1, and AP-2 binding sites are boxed.

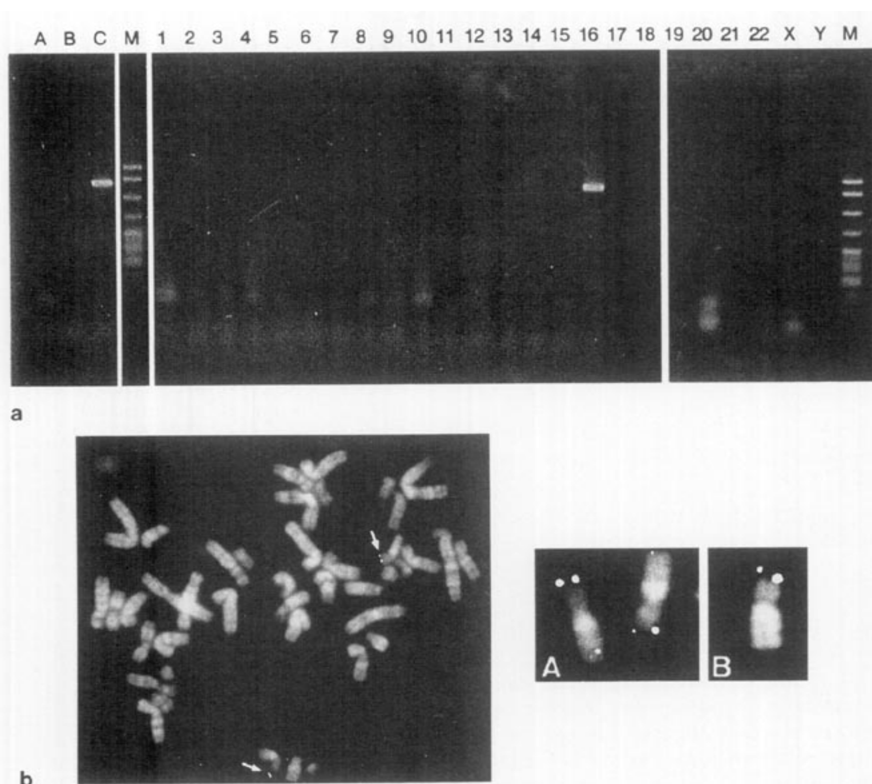

FIG. 4. (**a**) Assignment of human DCI gene to chromosome 16 by PCR amplification of DNA of NIGMS human/rodent somatic cell hybrid DNA of mapping panel 2. Oligonucleotide primers IsoC-1s and IsoC-2as were used (see Materials and Methods). The 507-bp PCR fragment (lanes **C** and **16**) covers exon V, intron 5, and exon VI. Lanes **A–C:** Templates were genomic DNAs from hamster, mouse, and human, respectively. Numbering of lanes 1–22, X, and Y refers to the human chromosomes present in the cell hybrid DNAs used as templates. **M,** marker. (**b**) Refined mapping of the human DCI to chromosome 16p13.3. The digoxigenin-labeled probe phDCIg-8 was hybridized to human metaphase chromosomes and visualized via antidigoxigenin conjugated to rhodamine. Chromosomes were counterstained with DAPI, and images of rhodamine or DAPI fluorescence were taken separately with a CCD camera. Both images were overlayed electronically to reveal the relative position of the signal to the chromosome. **A** and **B**, chromosomes from two different experiments with specific signals in 16p13.3.

expression remains to be established. Sequence analysis of the 3′-UTR of the DCI gene confirmed the respective region of the previously characterized DCI cDNA clone, which did not contain a typical polyadenylation signal.

Comparison of the human DCI gene with the mouse gene, characterized in this laboratory (Stoffel *et al.*, 1993), shows a high degree of structural similarities. The mouse gene also consists of seven exons and six introns spanning 14.9 kb. The exons I and II are larger in the human gene, whereas the exons III to VII are of identical size in both genes; beyond that, the exon/intron boundaries are perfectly conserved between the two species. The first two exons encode mainly the mitochondrial signal sequences of the preproteins 41 amino acid residues long in the human but only 28 in the mouse DCI. In contrast to these findings, the 5′-UTR and the putative promoter region of both genes share remarkably low homologies.

The results presented here together with the knowledge of the mouse gene provide the molecular basis to examine characteristics of mitochondrial β-oxidation of unsaturated fatty acids in humans. As an initial step, the importance of DCI for this pathway can be evaluated *in vivo* by generating a DCI-deficient mouse line using the technique of homologous recombination in mouse embryonic stem cells.

The knowledge of the human gene structure of an enzyme in the β-oxidation of unsaturated fatty acids may unravel the molecular basis of one of numerous clinical phenotypes that refer to an altered fatty acid oxidation. Furthermore, the DCI gene locus might be of importance as an additional marker for linkage studies of other congenital disorders residing in genes on chromosome 16p13.3.

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